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Dynamic metabolic control: towards precision engineering of metabolism

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Abstract

Advances in metabolic engineering have led to the synthesis of a wide variety of valuable chemicals in microorganisms. The key to commercializing these processes is the improvement of titer, productivity, yield, and robustness. Traditional approaches to enhancing production uses the “push-pull-block” strategy that modulates enzyme expression under static control. However, strains are often optimized for specific laboratory set-up and are sensitive to environmental fluctuations. Exposure to sub-optimal growth conditions during large-scale fermentation often reduces their production capacity. Moreover, static control of engineered pathways may imbalance cofactors or cause the accumulation of toxic intermediates, which imposes burden on the host and results in decreased production. To overcome these problems, the last decade has witnessed the emergence of a new technology that uses synthetic regulation to control heterologous pathways dynamically, in ways akin to regulatory networks found in nature. Here we review natural metabolic control strategies and recent developments in how they inspire the engineering of dynamically regulated pathways. We further discuss the challenges of designing and engineering dynamic control and highlight how model-based design can provide a powerful formalism to engineer dynamic control circuits, which together with the tools of synthetic biology, can work to enhance microbial production.

Keywords: Dynamic metabolic control, genetic circuits, biosensors, synthetic biology, model-based design

§ 1. Introduction

Microbial production of valuable chemicals provides an attractive alternative to petroleum-based synthesis routes. A wide variety of chemicals such as biofuels, pharmaceuticals, and nutraceuticals have been successfully produced in microbial hosts by assembling and optimizing metabolic pathways [71,47,48]. Typically, the expression of pathway enzymes is either constitutive or under the control of inducible promoters that are tuned to balance the pathway flux to maximize titers, productivities, and yields. Static overexpression of enzymes can impose a load onto the cell by competing for native resources from metabolism and draining resources such as ribosomes, ATPs and chaperones [23]. The extra load to the host cell also makes it challenging to dynamically balance resource allocation between cell growth and the engineered pathway. In addition, the obtained strains are often optimized under certain laboratory conditions and are not as robust in large bioreactors, where environmental fluctuations (e.g., nutrient concentration, temperature, dissolved oxygen, etc.) can subject cells to suboptimal conditions and lead to decreased production. Deviation from the optimal condition may divert carbon to byproducts or lead to the accumulation of toxic intermediates that attenuate cell growth [42]. Furthermore, engineered strains often suffer from stability issues where genetic mutations may arise during fermentation that deactivate the pathway activity. By comparison, natural cells maintain robust growth and withstand environmental fluctuations by dynamically adjusting cellular metabolism through complex regulatory networks. These regulatory networks govern the distribution of cellular resources and sustain homeostasis in fluctuating environments.

The study of how natural regulatory networks enable cells to grow robustly has been a focus in systems biology. Diverse regulation mechanisms have been identified to dynamically control metabolism in response to varying environmental conditions and intracellular metabolic status [29,39,30,16]. These mechanisms sense environmental signals such as nutrient

concentration, pH, and light, as well as intracellular metabolite concentrations and cell density. The sensed signals are then coupled to transcriptional, translational or post-translational processes to control protein expression or activities for efficient carbon usage. Taking the concept of dynamic regulation, synthetic biologists have designed genetic circuits to dynamically regulate engineered pathways for optimal biochemical production [73,24,18,76,37,22,70,68].

In this review we discuss dynamic control strategies found in nature and how they inspire engineering efforts to increase bioproduction, with a particular focus on the design of control architectures. We further discuss some of the key challenges to designing dynamic control for enhancing biochemical production and highlight the utility of mathematical models to help address these. We conclude with an outlook that integrating design principles learned from natural control systems and model-based design into the metabolic engineering workflow can facilitate the design of dynamic metabolic control, towards the development of robust and efficient microbial cell factories.

§ 2. Natural strategies for dynamic control of metabolism

Dynamic regulation of metabolic pathways is ubiquitous in nature. Spanning from simple microbes to multicellular animals, all forms of life depend on complex regulatory networks to coordinate metabolism to maintain cellular activity and adapt to environmental changes. To achieve this, cells use a variety of strategies that involve the interplay between DNAs, RNAs, regulatory proteins, enzymes and metabolites. Transcriptional regulation represents a significant level of control that is responsive to a wide variety of molecules and exhibits versatile regulatory architectures. In *Escherichia coli*, 577 interactions have been identified between transcription factors and their regulated operons [55], and this number is still growing. These complex

interactions are made up of network motifs with different architectures that give rise to different functions [1].

One major function of dynamic regulation is to allocate resources efficiently. This is mostly achieved by transcriptional control to avoid high cost of protein synthesis. At the transcriptional level, the expression of enzymes is often controlled by transcription factors that can sense either an intermediate or product of a pathway, generating different regulation architectures. For example, in the lysine biosynthesis pathway in *Saccharomyces cerevisiae*, the transcription factor Lys14 is activated by an intermediate alpha aminoadipate 6-semialdehyde (α AAS), which activates all the seven genes in the pathway. Similarly, enzymes in the arginine biosynthesis pathway of *E. coli* are repressed by ArgR, which is in turn activated by the end product arginine. Experimental analyses and cost-benefit models for enzyme expression have uncovered links between regulatory architecture and the timing of gene expression in unbranched pathways [44,15,72,17], revealing unique patterns of timing and **promoter activity** for efficient enzyme expression.

In addition to the transcriptional level, many cellular activities are modulated at the translational and post-translational levels, and oftentimes interplay among them. Translational regulation, usually through controlling translation initiation rate or mRNA stability, only respond to a small number of metabolites due to the limited chemical diversity of nucleic acids. Post translational regulation is abundant in metabolic pathways and controls enzyme activities in response to environmental stimuli or metabolite concentrations. For example, enzymes in *E. coli* central metabolism are heavily regulated at the post-translational level to tightly maintain constant metabolic flux under small environmental perturbations [50]. In addition, product allosteric inhibition of the first enzyme in metabolic pathways is commonly observed to rapidly turn down the metabolic flux through the pathway, allowing for immediate saving on carbon

usage. By comparison, transcriptional or translational regulation, though responding at a slower time scale due to slow protein synthesis and dilution, can drastically shift the distribution of metabolic flux and enable cells to save resources in the long run. Among different levels of regulation, transcriptional regulation offers a variety of traits desirable for engineering applications, including versatility in regulation architecture, chemical diversity of the sensed molecules, and tunability of the regulatory parameters. Indeed, transcriptional regulation is the most widely used control in metabolic engineering. Overall, understanding natural regulatory mechanisms provides us a wide variety of tools and design principles to develop synthetic dynamic control, which can be applied in metabolic engineering [36].

§ 3. Engineered strategies for dynamic control of metabolism

A synthetic dynamic control circuit typically consists of a biosensor and a genetic controller. The application of biosensors [75,39,35,74] and genetic control circuits [58,10] have been extensively reviewed. A variety of signals can be **detected to sense cellular growth status or a change in the environment**, such as intracellular metabolites, quorum signal molecules (AHLs), exogenous stimuli (inducers and lights), **and** environmental signals (pH, oxygen, and temperature). These signals can be used to repress or activate enzyme expression and thus regulate flux of a pathway. One primary design objective for dynamic control of metabolic flux is to balance the growth of the cell and production of the target molecule. Next, we discuss different types of design strategies from input signals to output regulations that attempt to address this objective.

The basic method to dynamically regulate the flux distribution is adding exogenous inducers or nutrients at a time point during fermentation (Fig. 1a). Xie et al. constructed a

glucose-dependent regulatory system in *S. cerevisiae* to control the flux from branch point farnesyl diphosphate (FPP) to ergosterol biosynthesis (an essential component in yeast membrane) or to the carotenoid pathway [69]. Squalene synthase (*erg9*), the first gene from FPP to ergosterol pathway, was placed under the HXT1 promoter, which was induced at high glucose concentration, while the carotenoid pathway was controlled by glucose-repressible GAL promoters so that the production pathway was turned on after glucose was partially replaced by glycerol as an alternative carbon source. Dynamic regulation by exogenous inducers is straightforward and effective, but is expensive, can cost the environment and required manual intervention. These problems can be overcome by introducing feedback control of enzyme expression to respond to signals produced by the cell itself.

An example of autonomous control is that on growth flux through negative feedback by a quorum sensing (QS) system (Fig. 1b). Soma and Hanai employed a QS system to autonomously redirect acetyl-CoA from the TCA cycle to the isopropanol pathway at a given cell density [60]. In a recent application, QS was used to downregulate phosphofructokinase-1 (*pfk-1*) in the upper glycolysis pathway [24]. Lower *pfk-1* activity channeled more carbon flux from the interconverting branch points G6P and F6P to the glucaric acid pathway, thus turning on product synthesis while inhibiting cell growth.

One key function of dynamic control in a biosynthetic pathway is to avoid accumulation of toxic intermediates or overexpression of toxic enzymes. Inhibiting an upstream pathway that generates the toxic intermediate and activating a downstream pathway that converts it are common control strategies (Fig. 1c). One of the pioneering works in dynamic pathway regulation was demonstrated for biodiesel production from free fatty acids [73]. In the pathway, accumulation of two intermediates ethanol and acyl-CoA is harmful to cell growth. The authors developed a dynamic regulatory system to activate ethanol production and the conversion of

ethanol and acyl-CoAs to final products only when fatty acyl-CoAs are sufficient. In another example, promoters responsive to FPP (toxic to cell) accumulation were used to repress the mevalonate pathway that produces FPP and to activate amorphaadiene synthase that consumes FPP. Such regulatory topology dynamically stabilized the FPP concentration below its toxic level, while increasing amorphaadiene production [18]. Similar control topologies can be constructed using transcription-factor-based sensors as demonstrated in the fatty acid pathway to optimize cellular malonyl-CoA pool [70]. In addition, synthetic inverters can be used to switch regulation between repression and activation, achieving a desired control topology [37].

Dynamic regulation can also be designed by sensing **signals to indicate** growth **status** and using **them** to regulate metabolite production. In one of the first examples of dynamic regulation, acetyl phosphate served as the signal for excess glycolytic flux to regulate the rate-limiting enzymes in lycopene pathway [22] (Fig. 1d). Recently, a biosynthetic pathway was controlled by a two-layered circuit, which acted as an AND gate that senses both the cellular growth status and the pathway precursor availability [38]. The first enzymatic step was not turned on until stationary phase and downstream steps were activated by the intermediate from the first step (Fig. 1e), which reduces burden from the engineered metabolic pathway. Synthetic control can also be designed to sense production flux and regulate growth (Fig. 1f). Xiao et al. described a strategy that uses metabolite product to activate cell growth via expression of an antibiotic pump, TetA [68]. This ensured that high producing cells would tolerate the antibiotic treatment, and thus facilitated the selection of high producing phenotypes at the population level. Without selection, a wide variation in biosynthetic performance was observed in the whole population. With selection, only the high-performing cells could survive, thus increasing total production.

Despite a growing number of success stories, engineering dynamic control remains extremely challenging. Current implementations require multiple iterations between construction

of part libraries, testing of different control architectures, and characterization of system performance. This lengthy design cycle is the result of multiple challenges that need to be addressed if the field is to move towards precision engineering of metabolism.

§ 4. Challenges for dynamic control and benefits of model-based design.

The capability to precisely control metabolism is key to optimizing complex and dynamic bioprocesses, and many challenges lie at different levels to apply dynamic control at an industrial scale. In large fermenters the level of intracellular metabolites and the environmental conditions can vary dramatically under different fermentation conditions. In addition, biosensors that work in model organisms may not function in an industrial host simply because the sensor is out of range. Therefore it is crucial to tune the parts to ensure the functioning of the dynamic control. The number of possible dynamic control design architectures can also be quite large, with a choice of what to sense, which enzymatic steps to regulate, and how to control enzyme expression levels (activation/repression). In the combinatorial explosion of possible designs, it is extremely difficult to experimentally determine the optimal design to give maximum, robust production, with minimum attenuation to cell growth demands. In addition, in large fermenters the difference in the time scale of heat and mass transfer also poses the challenge of how to tune dynamic control to match the magnitude and timescale of such environmental changes. Third, the control circuits need to function robustly within a host during long periods of fermentation. Thus, a good understanding of the host-circuit interaction is needed to account for resource allocation in the host. Lastly, in large bioreactors there are often increased cell-to-cell variations, and the challenge is how to

control the product distribution to shift the population to achieve higher percentage of high-producers.

In summary, current challenges for dynamic control **include** the construction **and tuning** of genetic parts, the assembly of parts into functional circuits, the interplay between circuit and host, and the control of population diversity (Fig. 2). Mathematical modelling is an ideal framework to integrate different design layers and explore the design space in a rational manner. Next, we discuss some of the key challenges ahead and outline how modelling can help overcome them.

§ 4.1. Construction of tunable parts

Metabolite biosensors are a key component of dynamic metabolic control. Their function is to control the expression of pathway enzymes in response to metabolic signals such as the concentrations of metabolic intermediates or other physicochemical cues. **Bioproduction growth conditions may shift signal concentrations to ranges that fall beyond the biosensor inducing threshold, impairing dynamic control and resulting in a effective static setting. It is therefore essential to be able to design and tune biosensor function to ensure its successful application, especially in industrially relevant conditions. In particular, biosensor function need to be designed to respond with the appropriate sensitivity, such as an all-or-none or graded response, cover a broad response dynamic range, and to reliably sense signal concentration and actuate the response at the right signal threshold, as appropriate for the growth conditions. Biosensor function can be captured in the** dose-response curve, which relates the concentration of the sensed metabolite to the enzyme expression (Fig. 2) **and its shape can be modified through experimentally** tunable parameters such as the metabolite binding affinity [Saeki et al (2015)] or

the sequence of target promoters [40]. Some of the successful implementations of dynamic control have demonstrated that tuning the biosensor dose-response curve can affect performance significantly and increase production [37,70,68]. The question of how to design dynamic control is thus critical to developing production strains, especially for application in industrial settings. Much work has focused on developing new biosensors, but the precise calibration of their dose-response curve remains poorly understood [3] and leads to lengthy iterations between biosensor construction and characterization.

Common biosensors in dynamic control are transcriptional riboswitches [7] and transcription factors [75]. Progress in RNA engineering has led to a growing number of riboswitches that respond to specific metabolites [66,26]. Studies have shown that RNA sequences shape the sensitivity and threshold of riboswitch dose-response curves [52,5], yet the precise tuning of riboswitch function remains a significant challenge. Computational methods have proven powerful for the design of RNA devices [13] and mathematical modelling has revealed insights on the tunability of the riboswitch function in terms of biophysical parameters [6]. Integration of sequence design algorithms with mathematical models may facilitate the discovery of new metabolite-responsive riboswitches, and thus expand the repertoire of pathways in which dynamic control can be used [7].

In the case of transcription factors, dose-response curves can be tuned with promoter engineering [40] or protein engineering to modify metabolite binding kinetics [Saeki et al (2015),63]. There are many natural transcription factors that respond to specific metabolites in their native host, which can be repurposed as biosensors in a production host of interest. Detailed biophysical models have revealed relations between sequence-dependent promoter binding affinities and protein expression [9]. Moreover, mathematical models have uncovered fundamental design constraints of dose-response curves, and together with experimental

validation, revealed a strategy for orthogonal control of biosensor dynamic range and threshold [40], an essential criteria for free design of biosensor function, and in turn real-world application.

§ 4.2. Assembling parts to design control circuits

Tunable parts provide the fundamental building block for the assembly of control circuits that will help to achieve an increased and robust production. Dynamic control circuits must achieve multiple design objectives simultaneously [46], where the goal is to construct control circuits that adapt pathway activity to varying bioreactor conditions, ensuring efficient expression of enzymes, minimizing the impact of pathway bottlenecks or accumulation of toxic intermediates, and ultimately maximize biochemical yield, titer or flux rate for economically viable production at industrial scales. Achieving all these objectives demands the availability of a wide repertoire of control circuit architectures, and the problem is the freedom in designing the architecture. In reality, architecture design is severely constrained by what can be sensed and the nature of the interaction between the metabolite-biosensor and biosensor-DNA (inhibitory or activatory) [75]. A metabolite that is indicative of the shift in condition and can be sensed is chosen, but its position, whether it is an end product or intermediate of the production pathway, can constrain the nature of the control [17]. Moreover, the choice of transcription factor sensing the chosen metabolite signal may not function as a dual regulator, only activating or inhibiting gene expression, further constraining the control design space. Key questions for architecture design thus are which pathway metabolite should be sensed, which enzymatic steps to implement dynamic control on and the nature of the controls (inhibit or activate enzyme expression). Mathematical modelling can be a powerful tool to explore such design space and assess performance of architectures that would otherwise be infeasible or too costly to test experimentally.

Unlike in static control, where genome-scale models can be used for strain design [11,57], model-based approaches for dynamic control are still in early stages. Mathematical models have revealed design principles to improve biofuel production through control of efflux pumps [21] and have provided conditions on the parameter design space to avoid accumulation of toxic intermediates [46]. Genome-scale models have been employed to determine which enzymes to control, which when coupled with dynamic modelling showed higher production as compared to static control [2]. A particularly promising use for modelling is the exploration of circuit architectures. Models have been used to search for architectures that efficiently trade-off production flux against toxicity effects by metabolic intermediates [61], to explore circuit architectures that function robustly in the face of environmental or genetic perturbations [25], or to discover new useful architectures, such as a bistable metabolic switch that filters out fluctuations in nutrient availability [43].

Control engineering has been tremendously successful in designing regulation systems for diverse disciplines such as aerospace, bioprocessing, and information technologies [4]. Principles from control engineering have gained ground in synthetic biology [19] and optimal control ideas have revealed design principles in natural metabolic systems [65,72], but their broader application to dynamic pathway control remains less explored. A potential area for future development is the use of mathematical optimization for circuit design [53] coupled with detailed kinetic models of metabolism [32,49,41,14,27] to maximize production (yield, titer or product flux rate). The optimization of control can be programmed to scan over circuit architecture and parameters simultaneously, but for increases in the control circuit detail and size exploration of the design space is quickly plagued by the curse of dimensionality. The trade-off between circuit size and computation time needs to be considered, and the development of more scalable optimization methods poses a challenge and opportunity for further research.

§ 4.3. Host-circuit interactions

As metabolic pathways and control circuits become larger and more complex, their footprint on their host can become a major limiting factor on function. Engineered systems draw resources from the host, which can disrupt homeostasis and cause growth defects that lead to poor or even altered functionality [62,12]. A key source for host-circuit interactions is the competition for cellular resources such as ribosomes, RNA polymerases, and amino acid pools [8]. This competition affects cell growth and ultimately may result in **an impaired circuit function, and in turn make for a suboptimal metabolic production that is economically impracticable for industrial level production.**

Mathematical models can give a systems-level understanding of the relationship between circuit function and the physiology of the host where they reside. To this end, Weiße and colleagues developed a mechanistic model for bacterial growth, based on a coarse-grained partition of the proteome and its interaction through metabolism, transcription and translation [67]. The model predicts growth defects caused by gene circuits and provides a quantitative platform to assess the impact of growth defects on circuit function. A recent extension to this work includes more detailed mechanisms of the different host-circuit crosstalks and proved useful for circuit design [34]. Models for host-circuit interactions do not yet allow the inclusion of dynamic pathway control, but the use of dynamic control to manage host load and increase production is promising, especially in light of recent evidence showing that feedback control can mitigate the impact of resource coupling [56].

§ 4.4. Control of population heterogeneity

Phenotypic heterogeneity is ubiquitous in cellular populations. In microbes, heterogeneity has been extensively studied as a product of stochasticity in gene expression and the resulting

variation in protein levels [51], and recent work has focused on variability on metabolic phenotypes and growth [59,31,28]. Though phenotypic variability in natural systems can serve as a population survival strategy, variability amongst strains engineered for production can lead to suboptimal performance. Phenotypic variability may also result from fluctuations in growth conditions, and in scaling-up to industrial level production inhomogeneities in growth media can become exaggerated. In strains engineered for chemical production, phenotypic variability manifests itself as wide distributions of metabolic production [54,20]. Such variability has been exploited to increase production by designing control that couples the concentration of product to growth, and thereby selects for high producers [68].

Mathematical modelling can provide novel insights on the sources and control of metabolic variability. For example, the integration of genome-scale models with single-cell proteomics datasets revealed the emergence of a bimodal growth distribution in *E. coli* [33], and the emergence of bimodal phenotypes was also explored with dynamic models [64,31]. A seminal stochastic modelling work on enzymatic reactions revealed conditions for a dynamic control circuit to amplify or attenuate the variability of a metabolic product [45].

It is worth noting that the emergence of mutants and genotypic heterogeneity pose a significant problem for long term biochemical production, and can plague the implementation of production strains at industrial scales. Over long time scales of fermentation mutations may impair the control circuit or result in the emergence of non-producing, faster growing strains that will dilute out production strains. Though there is some understanding that very low expression of DNA repair mechanisms, such as demethylation by N-Ada protein, can lead to the emergence of genotypic heterogeneity [(Uphoff et al (2016))], it is unclear how to control for it. This is a key area for future development to help sustain long-term bioproduction in industrial settings.

§ 5. Final remarks

Dynamic control of metabolism is a powerful mechanism for cells to survive and adapt to environmental perturbations. In natural systems, dynamic control shifts metabolic activity between various operating regimes. Metabolic engineering can harness similar control strategies to increase production in varying and often unpredictable bioreactor conditions. In this paper we outlined some of the natural strategies for dynamic control together with recent successful implementations on metabolic production pathways.

Dynamic control has vast potential to enhance production at the industrial scale, enabling autonomous control of pathway activity without the cost of inducers and auto-adapting production and cellular demands according to fluctuating or changing fermentation conditions. Challenges for this technology are manifold and cover several layers of complexity, from tunable control parts, to functional circuits, accounting for host physiology and demands of the cell, and sustaining production in the face of phenotypic and emergence of genotypic heterogeneity. In this paper we discussed the challenges at these levels and how they affect the application of strains engineered with dynamic control to industrial scale bioproduction. Although a few recent studies have demonstrated the computation-guided tuning of biosensor response, the reliable determination of the intracellular metabolite concentration remains a challenge to providing accurate inputs to the model. In addition, the application of dynamic control in industrially relevant hosts has been limited, which entails tools and efforts to transfer the technology into those hosts (cite lysine paper). Robust controls need to use the host resources efficiently and optimize the balance between growth and production. This is a challenging objective to achieve, and one where the metabolic engineering community can learn valuable lessons from natural systems. Systems biology has revealed fundamental design principles by reverse-engineering the regulation of natural metabolic systems, thanks to the combination of mathematical modelling

and wet-lab experimentation. Natural design principles and model-based methods integrated into the metabolic engineering workflow could institute the forward-engineering of control circuits and hail a new era in which dynamic control becomes the key technology for optimizing chemical production.

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Figures

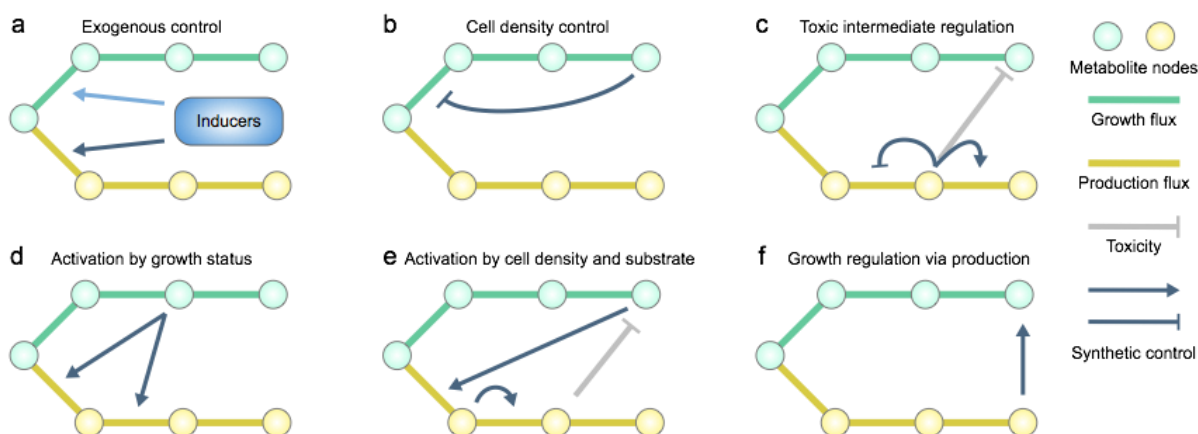


Figure 1. Engineered control strategies of metabolic pathway in bioproduction. To abstract the designs of different dynamic control strategies, we represent cell growth and product biosynthesis as two linear fluxes that branch from the same precursor metabolite, where growth encompasses fluxes towards essential metabolites (e.g. TCA cycle, amino acid biosynthesis, nucleotide biosynthesis, membrane biosynthesis). (a) Using inducers to control flux from the branch point [69]. (b) Using QS systems to control growth flux by negative feedback circuits [24,60]. (c) Using metabolite-responsive regulators to control toxic intermediate levels [73,18,37,70]. (d) Using growth flux to activate the production pathway [22]. (e) Using metabolite levels and growth status, which accounts for toxic effects, to regulate the production pathway [38]. (f) Using product level to control survival of the cells [68].

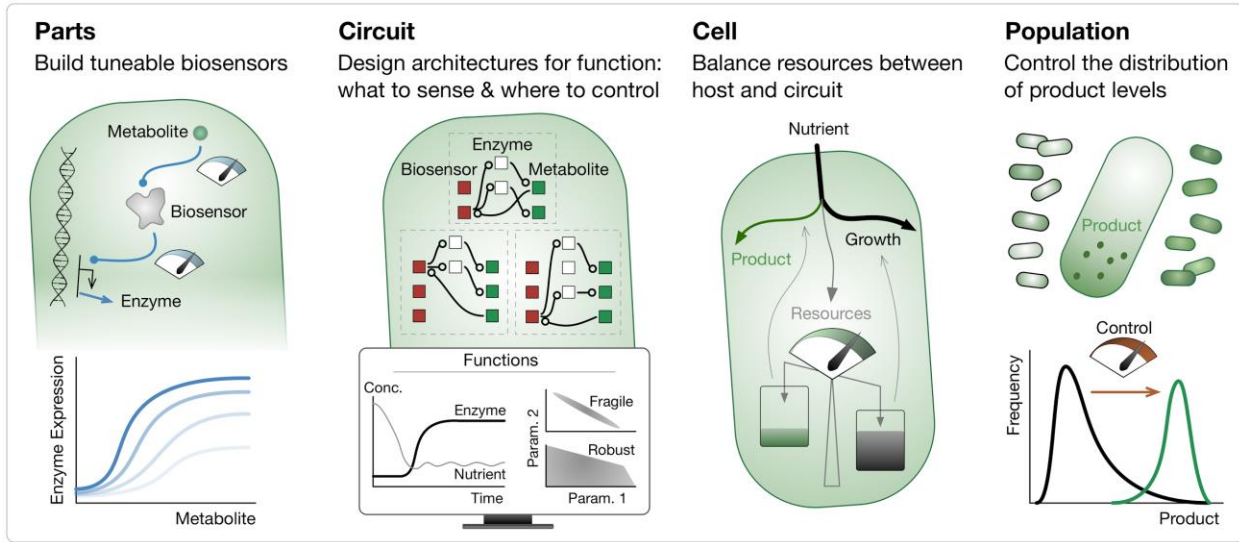


Figure 2. Challenges for designing dynamic control circuits at various levels. These challenges include how to tune parts to obtain desired dose-response functions, **when control is actuated by riboswitches [6,52] or transcription factors [40,63]**; how regulatory architectures affect dynamics [17,46,61] and robustness [43], **as learned from models of natural control systems**; how to balance limited resources between growth and production, **studied theoretically**

[34,67]; and how to control cell-cell heterogeneity for sustainable and efficient production, studied theoretically [45] and experimentally [68].